Research Paper:

Human Olfactory Ecto-mesenchymal Stem Cells Displaying Schwann-cell-like Phenotypes and Promoting Neurite Outgrowth *in Vitro*

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ABSTRACT

Introduction: Strategies of Schwann cell (SC) transplantation for regeneration of peripheral nerve injury involve many limitations. Stem cells can be used as alternative cell source for differentiation into Schwann cells. Given the high potential of neural crest-derived stem cells for the generation of multiple cell lineages, in this research, we considered whether olfactory ectomesenchymal stem cells (OE-MSCs) derived from neural crest can spontaneously differentiate into SC lineage

Methods: OE-MSCs were isolated from human nasal mucosa and characterized by the mesenchymal and neural crest markers. The cells were cultured in glial growth factors-free medium and further investigated in terms of the phenotypic and functional properties.

Results: Immunocytochemical staining and real-time PCR analysis indicated that the cultured OE-MSCs expressed SCs markers, SOX10, p75, S100, GFAP and MBP, differentiation indicative. It was found that the cells could secrete neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Furthermore, after co-cultured with PC12, the mean neurite length was enhanced by OE-MSCs.

Conclusion: The findings indicated that OE-MSCs could be differentiated spontaneously into SC-like phenotypes, suggesting their applications for transplantation in peripheral nerve injuries.

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Highlights

- OE-MSCs from human nasal mucosa showed the significant mitotic activity in comparison to ASCs and SCs.
- hOE-MSCs had tendency to differentiate spontaneously into SC-like phenotypes.
- OE-MSCs could secrete neurotrophic factors and promote neurite outgrowth in vitro.
- hOE-MSCs as a valuable source of autologous stem cells had a potential for the transplantation in PNIs.

Plain Language Summary

In this study, spontaneously differentiation of olfactory ectomesenchymal stem cells (OE-MSCs) derived from neural crest into SC lineage was considered. After isolation and culture of OE-MSCs in glial growth factors-free medium, they were investigated in terms of glial markers expression, secretion of neurotrophic factors and promotion of neurite outgrowth using immunocytochemical staining, real-time PCR analysis and Elisa test. The findings of the present study indicated tendency of hOE-MSCs to spontaneous differentiation into SCs-like cells and promotion of neurite outgrowth, therefore their applications for transplantation in peripheral nerve injuries were suggested.

1. Introduction

The application of cell-based therapies is one of the most promising strategies for treating peripheral nerve injuries, whereby trophic factors and cytokines are provided by engrafted cells (Bagher et al., 2015; Hussain et al., 2020). Schwann cells (SCs) as major myelin-forming cells and a key component in peripheral nerve tissue, play important roles in the process of nerve regeneration (López-Cebral et al., 2018). Following nerve injury, these cells proliferate rapidly and dedifferentiate while clearing the myelin debris (Jessen & Mirsky, 2019). SCs promote and direct axonal outgrowth from the proximal to distal neural ends by releasing various neurotrophic factors and extracellular matrix, forming the Büngner bands that act as longitudinal guidance channels (Hassanzadeh et al., 2021; Jessen & Mirsky, 2019; Zochodne et al, 2019). Since during large neural defects, SCs are inadequate, it is critical to supply a sufficient rate of SCs to repair the site (Lotfi et al, 2019). However, the supply of cultured autogenic SCs is limited by the lack of availability, donor site morbidity, and the low capacity to proliferate remarkably that delay the treatment. Thus, alternative cell sources that can differentiate into functional SCs are desirable (Petrova 2015; Resch et al, 2019).

Neural crest stem cells are known as a type of multipotent progenitor cells that give rise to various phenotypic lineages, including SCs. In the craniofacial region, these stem cells are found in some tissues, such as dental pulp (Haratizadeh et al., 2016; Pisciotta et al., 2020; Solis-Castro et al., 2020), periodontal ligament (Pisciotta et al., 2020), dermal papilla (Hunt et al., 2008), and nasal mucosa (Duan & Lu, 2015).

Olfactory ecto-mesenchymal stem cells (OE-MSCs) are a new population of neural-crest-derived stem cells (Duan & Lu, 2015) that can be obtained from the olfactory nasal mucosa niche using non-invasive techniques (Simorgh et al., 2021a; Simorgh, et al., 2021b). Ecto-mesenchymal stem cells (EMSCs) possess unique properties, including multilineage differentiation and self-renewal capacity, higher mitotic activity compared to mesenchymal stem cells isolated from other tissue sources especially bone marrow mesenchymal stem cells (BM-MSCs) (Delorme et al., 2010), immunoregulatory function (Rui et al., 2016), and the lack of apoptotic and tumorigenic activities (Alvites et al., 2020; Shafiee et al., 2011; Veron, et al., 2018). Moreover, it has been demonstrated that EMSCs have a higher potential compared to other sources of stem cells to secret neurotrophic factors and differentiate into different neurogenic cells, including SCs because of originating from the neural crest (NC) (Veron et al., 2018; Zhang et al., 2015). These stem cells are also known as Schwann progenitor cells and express SC markers, namely S100 and $p75^{NTR}$ (Chen et al., 2015). Therefore, they can be considered a good choice for cell therapy in peripheral nerve injuries. Recent research has shown that EMSCs are capable of spontaneously differentiating into various lineages, such as melanocyte (Paino et al., 2010), smooth muscle, and osteoblast lineages (Lin et al., 2006), whereas their differentiation into glial cell lineage has not been well understood. EMSCs differentiation into SC-like cells has

previously been represented in a specific glial differentiating medium (Nie et al., 2007; Zhang et al., 2015). In this research, for the first time, we aim to investigate the spontaneous differentiation of EMSCs from human olfactory mucosa into functional SC-like cells by examining SC-specific markers expression, neurotrophic factors secreted by OE-MSCs monolayer at culture media, and neurites outgrowth from PC12 cells on a co-culture assay.

2. Materials and Methods

Isolation and culture of human olfactory ecto-mesenchymal stem cells

Human nasal mucosa cells were obtained using a modified technique as previously described (Karimi et al, 2021). Nasal mucosa biopsies were collected from individuals in the age range of 20 to 50 years, undergoing nasal surgery at Iran University Hospital Research Center. In brief, after local anesthesia, the biopsy was performed from the nasal superior conchae. The samples were immediately washed with phosphate-buffered saline (PBS) and placed in DMEM/Ham's F12 (Gibco, USA) supplemented with 1% penicillin/streptomycin (Pen/Strep; Gibco, USA). The lamina propria was isolated under a dissecting microscope and incubated for 40 min at 37°C in dispase II solution (2.4 U/mL). Then, the suspension of the cells was placed into flasks and cultured in a growth medium, DMEM/F12 supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% Pen/Strep at 37°C with 5% CO_2. The media was changed every 72 h and when the adherent cells reached 80% confluence, they were passaged. The cells in the fourth passage were used for the study.

Characterization of human olfactory ecto-mesenchymal stem cells

The cells were confirmed by morphological assessment, flow cytometry analysis for CD105, CD90, and CD73 as positive markers, and CD45 and CD34 as negative markers, and immunofluorescence staining for examining the NC markers, nestin and vimentin. In addition, the multilineage differentiation capacity of OE-MSCs was assessed by the induction into osteogenic and adipogenic lineages.

Cell proliferation assay

The MTT assay was done to determine the growth rate of human OE-MSCs and compare that with human adipose tissue-derived stem cells (ASCs) as an identified and accessible source from human MSCs that can be differentiated into SCs (Fu et al., 2016; Kinghamet al., 2007). Moreover, the viability rate of OE-MSCs was separately compared to human SCs. The cells at passage 4 were plated at a density of 2×10^3 cells/well in a 96-well plate for 1, 4, and 7 days. Then, the MTT solution was added to the cells and incubated for 4 h. The insoluble formazan crystals were dissolved using dimethyl sulfoxide and the absorbance was measured at 570 nm.

Evaluation of schwann-cell-like phenotypes in human olfactory ecto-mesenchymal stem cells

To evaluate the potential for spontaneous differentiation of OE-MSCs into SC-like cells, human OE-MSCs were cultured in a medium, DMEM/F12 supplemented with 10% FBS, and 1% Pen/Strep for 7 days (Chen et al., 2015). Then, the expression of SC-specific markers, genes, and secreted proteins was tested through immunocytochemistry, quantitative real-time polymerase chain reaction (qRT-PCR), and enzyme-linked immunosorbent assay (ELISA), respectively.

Immunofluorescence assay

For immunostaining (Kingham et al., 2007), the cells were fixed in fresh 4% paraformaldehyde (PFA) for 15 to 20 min. After washing with PBS, the fixed cells were blocked with 10% goat serum for approximately 1 h. Then, primary antibodies, mouse monoclonal anti-S100 (1:400; Abcam), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200; Abcam), and mouse anti-myelin basic protein (MBP; 1:20; Sigma) were added, and the cells were incubated at 4°C overnight. The cells were treated with Texas Red goat anti-mouse, Alexa Fluor 488 goat anti-mouse, and FITC goat anti-rabbit (IgG) as secondary antibodies (1:500; Abcam), respectively, for 1 h in the dark at room temperature. Finally, cell nuclei were labeled with 4′,6′-diamidino-2-phenylindole dihydro-chloride (DAPI; Sigma) and evaluated by a fluorescence microscope. For the quantification of immunocytochemistry, the number of total cells and positively stained cells were counted.

Quantitative real-time polymerase chain reaction analysis

The total mRNA within human OE-MSCs was extracted using TRIzol. Then, isolated RNAs were used for cDNA synthesis by the reverse transcription kit (Fermentas, Germany). Real-time PCR was done using the SYBR Master Mix (Takara, Japan). The primer sequences are listed in Table 1.

Table 1. Primer sequences in real-time polymerase chain reaction

Target Gene	Primer Sequence (5'-3')
SOX10	F: GATTCAGGCTCCGTCCA R: TCGCAAAGAGTCCAACG
GFAP	F: GCAGACCTTCTCCAACCTG R: ACTCCTTAATGACCTCTCCATC
S100	F: AGCACACCCTGAGCAAGA R: TCACCTCCTGGTCCTTGT
P75	F: GAAGGGAGCACTGGCG R: TGATGACACAGTTCACCACTC
MBP	F: ACCCCGTAGTCCATTCTTC R: ACTCCCTTGAATCCCTTGTG
β-actin	F: CTTCCTTCCTGGGCATG R: GTCTTTGCGGATGTCCAC

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The DNA amplification was performed under the following conditions: after the pre-degeneration at 95°C for 2 min, 40 cycles in two steps were performed at 95°C for 5 sec and 66°C for 30 sec. The reactions were done in triplicate. The relative expression of each mRNA was calculated through the ΔΔCT method and normalized to that of β-actin as the housekeeping gene.

Human olfactory ecto-mesenchymal stem-cells-secreted neurotrophic factors

The released neurotrophins, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) in the OE-MSCs-conditioned medium were analyzed by an ELISA test (Peng et al., 2011). The conditioned media from the human SCs were purchased from the Stem Cell Research Institute (Bonyakhteh, Iran) and the base medium was used as the control group. To collect the conditioned media, 1×10^4 cells/mL OE-MSCs and SCs were seeded on a 48-well plate and incubated overnight. After that, the media was replaced with DMDM/F12 plus 1% FBS and cultured for 72 h at 37°C and 5% CO_2. The conditioned media was collected and frozen at -80°C until assessment. The protein levels of NGF and BDNF were measured via the ELISA, human β-NGF DuoSet, and human BDNF Quantikine ELISA kits (R&D Systems Inc., USA) and measured at 450 nm absorbance according to the manufacturer's protocol.

Co-culture of human olfactory ecto-mesenchymal stem cells with PC12 cells

To investigate the function of human OE-MSCs on neurites outgrowth, we examined the co-culture of the OE-MSCs with rat pheochromocytoma (PC12) cells applied as an *in vitro* model for investigating neuronal differentiation (Xue et al., 2017). The PC12 cells were seeded at a density of 500 cells/well on the monolayer of human OE-MSCs. After 24 h incubation in DMEM/F12 plus 10% FBS, it was switched to a medium supplemented with 1% FBS and 50 ng/mL NGF (Sigma) for 5 days. The media was changed every 72 h. PC12 was grown alone on a tissue culture plate and PC12 seeded with human SCs was used as control cultures.

Neurite outgrowth

The behavior of neurites extending from PC12 was evaluated by immunostaining with anti-β III tubulin antibody (1:200; Millipore) and Alexa Fluor 488 goat anti-mouse IgG (1:500; Abcam) as a secondary antibody and was imaged under a fluorescence microscope. For the quantification of neurite outgrowth. Images of 10-15 cells from at least 5 randomly selected fields per well were analyzed. Neurites in each field were measured from soma to the end of neurite using the ImageJ software and then averaged. Subsequently, the maximal neurite length in each field was calculated.

Statistical analysis

The data were expressed as a Mean±SE. To analyze the data, we used GraphPad Prism software, version 7. The statistical analysis was done using one-way and two-way analysis of variance (ANOVA) to compare the differences between groups. The $P<0.05$ was considered a statistically significant difference.

3. Result

Identification of human olfactory ecto-mesenchymal stem cells originated from neural crest

Morphologically, olfactory stem cells appeared as fibroblast-like cells and spindle-shaped. Here, these cells proliferated rapidly and after a short time, they were harvested at 80% confluency (Figure 1 A). The multipotency capacity of OE-MSCs was confirmed through differentiation toward two lineages, namely osteogenesis and adipogenesis by Alizarin Red S and Oil Red-O staining, respectively (Figure 1 B, C). Immunofluorescent staining revealed that almost all of the human OE-MSCs expressed nestin and vimentin (Figure 2 A). Moreover, flow cytometry analysis showed that the isolated human OE-MSCs expressed CD105 (97.18%), CD73 (98.12%), and CD90 (98.98%), while they were negative for hematopoietic markers CD45 and CD34 (Figure 2 B).

Proliferation rate of human olfactory ecto-mesenchymal stem cells

For the MTT assay, OE-MSCs, ASCs, and SCs isolated from human tissues at passage 4 were plated at a density of 2×10^3 cells/well. As shown in Figure 3 A, the proliferation rate of MSCs taken from nasal mucosa was significantly higher than those from the adipose tissue. The remarkable growth of OE-MSCs started at early hours, and after 7 days displayed an almost 3-fold increase compared to ASCs (Figure 3 A). The same result was also obtained when the growth rate of OE-MSCs was compared to that of human SCs. (Figure 3 B). MTT showed that the expansion of OE-MSCs was more rapid compared to human SCs on all evaluated days, suggesting the lower proliferation ability of human SCs (****P<0.0001).

Evaluation of schwann-cell-like phenotypes in cultured human olfactory ecto-mesenchymal stem cells

One week after the OE-MSCs culture, the expression of SC-specific markers was tested. The immunofluorescent staining showed that human OE-MSCs were positive for markers S100 (86.03%±9.41%), MBP (39.22%±3.66%), and GFAP (39.8%±8.4%) (Figure 4 A). To confirm the results, RT-qPCR was performed on human OE-MSCs (Figure 4 B). The levels of mRNA SOX10, S100, p75, GFAP, and MBP were analyzed, and the results showed the expression of all genes in human OE-MSCs. However, the SOX10 marker showed the highest expression in between genes, and the level of the S100 marker was significantly higher than that of other mRNAs (***P<0.001, *P<0.05). No remarkable differences in levels of p75, MBP, and GFAP mRNAs were found in human OE-MSCs.

Functional properties of cultured human olfactory ecto-mesenchymal stem cells

To evaluate the similarity of OE-MSCs function with that of SC-like, the levels of secreted neurotrophins, BDNF, and NGF in the OE-MSCs-conditioned medium were detected by the ELISA quantification (Figure 5 A, B). The results showed that OE-MSCs secreted detectable levels of BDNF and NGF (***P<0.001). Also, there was no statistically significant difference between OE-MSCs and SCs in levels of both neurotrophic factors. The ability of OE-MSCs to promote the extension of neurites was revealed by assessing their interaction with PC12 cells after 6 days (Figure 6). The analysis and quantification of co-culture images showed that neurites of PC12 were more elongated in the presence of OE-MSCs and SCs, and neurite extension increased

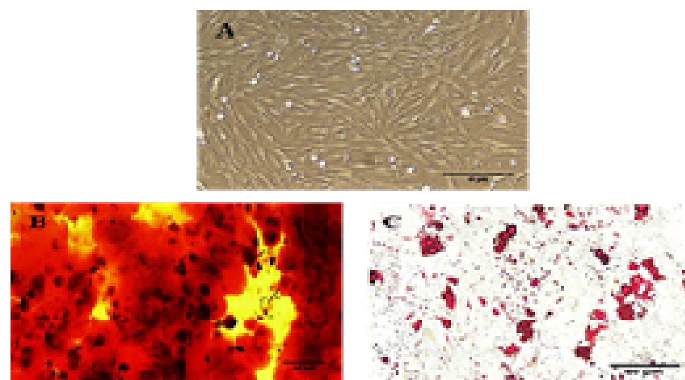

Figure 1. Characterization of human olfactory ecto-mesenchymal stem cells

A) The olfactory ecto-mesenchymal stem cells at the fourth passage formed a monolayer of fibroblast-like cells under phase contrast, B) The differentiated olfactory ecto-mesenchymal stem cells into osteocytes stained with Alizarin Red S, C) The differentiated olfactory ecto-mesenchymal stem cells into adipocytes stained with Oil Red-O (scale bar=40 µm).

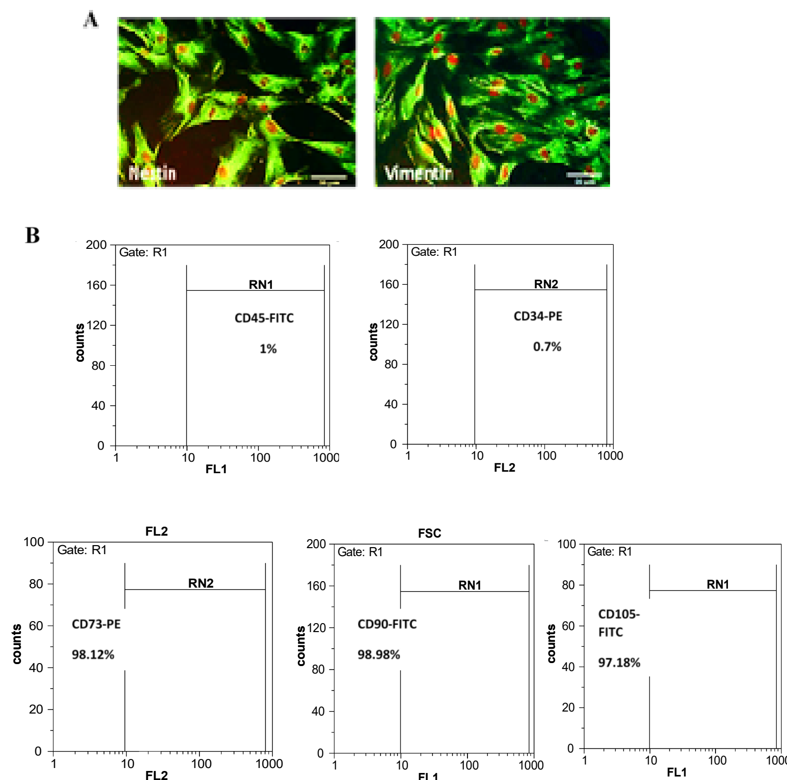

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Figure 2. Characterization of human olfactory ecto-mesenchymal stem cells by immunocytochemistry and flow cytometry analysis

A) The immunocytochemical staining showed that human olfactory ecto-mesenchymal stem cells expressed nestin and vimentin (scale bar=20 µm); B) The flow cytometric analysis indicated that the cells were positive for CD105, CD73, and CD90, and negative for CD45 and CD34.

significantly to 131.9±49.5 µm and 154.2±83.8 µm, respectively (**P<0.01, ***P<0.001) (Figure 6 B). In contrast, PC12 cultured alone on tissue culture plate has shown short neurites (87.9±38.2 µm). Likewise, the longest neurite length significantly increased to 214±83.8 µm, and 262.6±72.6 µm, in groups of co-culture with OE-MSCs and Schwann cells, respectively (*P<0.05, **P<0.01), compared to PC12 alone (139±26.5 µm) (Figure 6 C). There was also no significant difference between the co-cultured groups.

4. Discussion

Current cell transplantation strategies for facilitating remyelination and nerve regeneration face limited SCs expansion. Various types of stem cells can be considered promising alternatives to overcome these limitations (Jiang et al., 2017; Zaminy et al., 2013). Given the high potential of NC-derived stem cells as a source of autologous stem cells for the generation of glial cell types, recent studies have focused on ecto-mesenchymal stem cells originating from NC and their differentiation into SC-like cells (Nie et al., 2007). The results have shown that these stem cells differentiate to SC phenotypes under different differentiation conditions (Zhang et al., 2015). In the present study, spontaneous differentiation of EMSCs obtained from human nasal olfactory mucosa into SC-like cell phenotypes was investigated. The isolated human OE-MSCs were characterized in terms of having fibroblastic-like and spindle-shaped morphology, the expression of a set of ecto-mesenchymal stem cells-associated markers, and multipotent differentiation capability. Our experiments confirmed the three characteristics mentioned in human OE-MSCs, in line with previous studies on other EMSCs (Chen et al., 2015; Rui et al., 2016). The growth rate of OE-MSCs was determined and further compared with that of cells isolated from human adipose tissue and human SCs, separately. Both results showed that OE-MSCs proliferated significantly faster than ASCs and SCs.

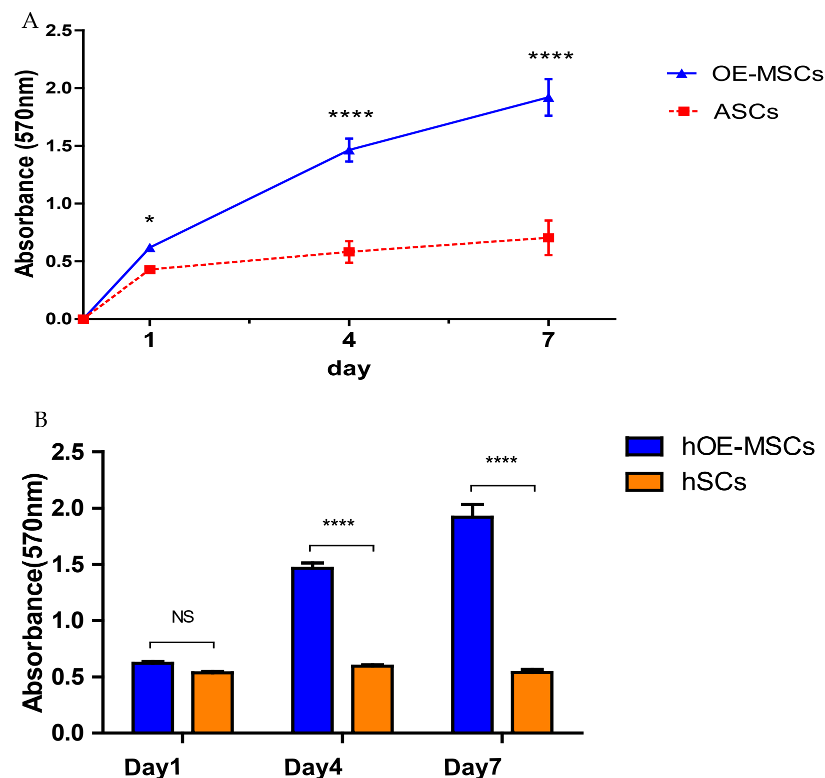

Figure 3. The proliferation of ecto-mesenchymal stem cells from human olfactory mucosa, mesenchymal stem cells from human adipose tissue, and human schwann cells

A) The MTT assay determined the growth rate of human olfactory ecto-mesenchymal stem cells (▲) and human adipose tissue-derived stem cells (▪); B) Human olfactory ecto-mesenchymal stem cells and human Schwann cells for 1, 4, and 7 days in absorbance (570 nm). *P<0.05, ****P<0.0001, NS: Not significant.

These findings were confirmed by Delorme and colleagues in a study that displayed a near 3-fold proliferation rate of OE-MSCs compared to that of bone marrow MSCs (Delorme et al., 2010), and also several studies that demonstrated poor expansion and prolonged *in vitro* culture of SCs compared to stem cells especially MSCs (Peng et al., 2011; Petrova, 2015). Given that a large number of cells are usually required in transplantation (Xue et al., 2017), the high proliferation of human OE-MSCs showed that these stem cells can be considered a reliable source for cell therapy. In this research, we found that OE-MSCs cultured without the presence of glial growth factors expressed typical markers of SCs. They secreted high levels of neurotrophins and enhanced the neurite outgrowth from PC12 cells in the co-culture system, displaying trophic effects and phenotypic characteristics similar to those of SCs (Lin et al., 2006).

Consistent with previous studies, our study showed that almost all OE-MSCs expressed NC markers, namely nestin and vimentin. Nestin and vimentin are intermediate filament proteins that can be used for the identification of neural stem cells (Biagiotti et al., 2006). Recently, the expression of nestin has been observed in MSCs from bone marrow and adipose tissue, and its importance has been demonstrated for the committed differentiation along the glial lineage (Caddick et al., 2006; Kingham et al., 2007). It has been reported that the potential of glial cell formation by adipose and bone marrow-derived MSCs may be due to the presence of NC-derived stem cells (Zhang et al., 2015). During the development, NC multipotent stem cells give rise to a variety of lineages, including SCs (Achilleos & Trainor, 2012; Mehrotra et al., 2020).

These cells transform to mature SCs (myelinating and non-myelinating SCs) through the generation of SC precursors (SCPs) and immature SCs (iSCs) (Jessen & R. Mirsky, 2019). The extensive changes take place in a set of phenotypic features, including the expression of genes and proteins at every stage of SC development that is used to identify a state of SCs. For instance, there was no expression of GFAP and S100 markers in SCPs, whereas they were upregulated in iSCs differentiated from SCPs (Mirsky et al., 2008; Woodhoo & Sommer, 2008).

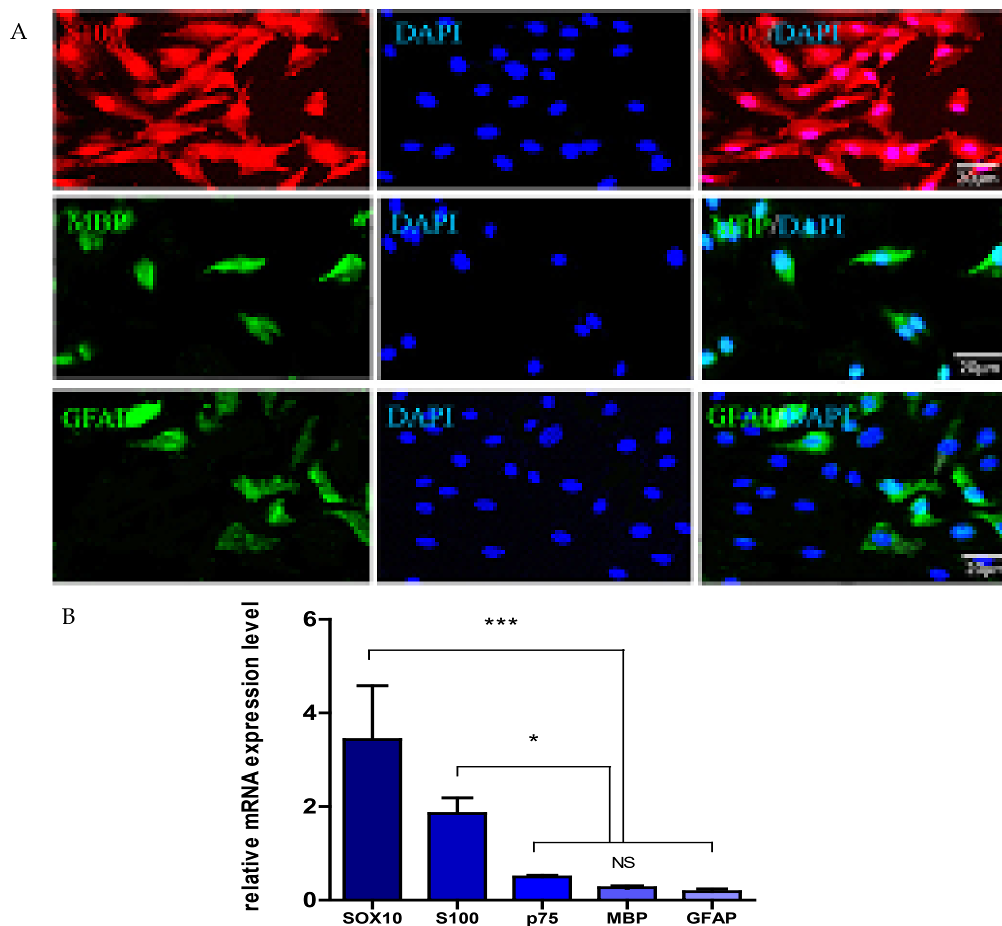

Figure 4. The expression of glial cell markers in human human olfactory ecto-mesenchymal stem cells NEUROSCIENCE

A) The immunocytochemical staining showed S100, MBP, and GFAP in olfactory ecto-mesenchymal stem cells cultured in nerve growth factors-free media (scale bar=20 µm), B) The real-time PCR analysis showed mRNA levels of Schwann cells at different states in olfactory ecto-mesenchymal stem cells differentiated in nerve growth factor-free media; S100 and SOX10 were significantly higher than other genes; *P<0.05 and ***P<0.001.

Also myelinating SCs expressed a range of myelin-associated genes, such as MBP. Nevertheless, some markers like p75NTR and SOX10 showed no significant change and presented in a whole of early developmental stages (Woodhoo & Sommer, 2008). Although it has been demonstrated that p75, S100, GFAP, and MBP, as glial markers, were expressed strongly in EMSCs differentiated to SCs phenotypes under induction medium (Nie et al., 2007; Zhang et al., 2015), the expression of some of them has been observed in EMCSs cultured at growth factor-free medium (Alvites et al., 2020; Chen et al., 2015; Zhang et al., 2015). In this study, immunofluorescent staining of spontaneously differentiated human OE-MSCs displayed that most cells were immune-positive for S100 and GFAP markers, and a few cells were GFAP and MBP-positive. This result was confirmed by the qRT-PCR analysis and showed the co-expression of SC-specific genes, comprising SOX10, S100, p75, MBP, and GFAP. However, the mRNA level of p75, MBP, and GFAP was significantly lower than that of other genes. In line with our study, a recent study showed that EMSCs from respiratory nasal mucosa could differentiate spontaneously into SC phenotype on a fibrin gel substrate (Chen et al., 2015).

In another research, spontaneous differentiation of EMSCs from dental papilla into mature melanocytes was demonstrated (Paino et al., 2010) and showed that some EMSCs of the first branchial arch could differentiate spontaneously into osteoblast and smooth muscle lineages, but not glial lineage (Lin et al., 2006). It has been also suggested that the fate of migrating pluripotent and uncommitted NC cells may be dictated by local factors in their environment (Deng et al., 2004; Liu et al., 2012). These cells and their derived-EMSCs after migration to the target tissues could obtain a degree of commitment

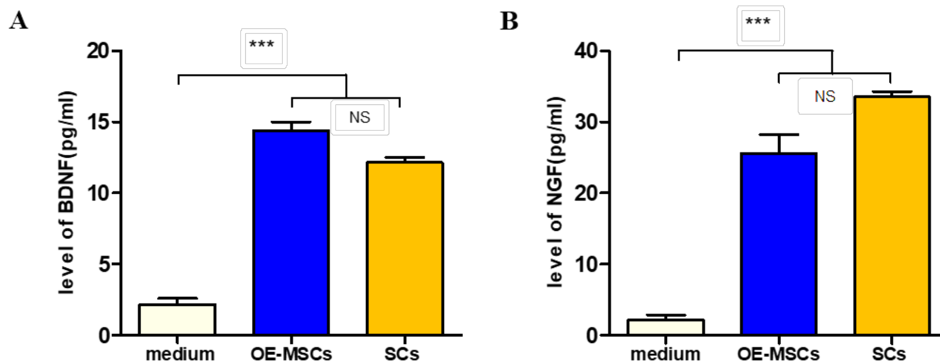

Figure 5. The neurotropic factor secreted from olfactory ecto-mesenchymal stem cells

A and B) Detectable levels of brain-derived neurotrophic factor and nerve growth factor (pg/mL) in human olfactory ecto-mesenchymal stem cells; *** P<0.001.

to be spontaneously promoted without various growth factors (Deng et al., 2004). It could be the reason that EMSCs isolated from different sites tend to differentiate spontaneously along specific lineages. In our study, these expressed markers showed that human OE-MSCs were similar to immature SCs.

The phenotypic characteristics are not usually enough data to justify whether the function of OE-MSCs is similar to SCs (Peng et al., 2011). As mentioned before, SCs dedifferentiate and convert into an immature state in response to peripheral nerve injury (Mirsky et al., 2008). These SCs proliferate rapidly and secrete various neurotrophic factors that promote axonal regrowth (Boerboom et al., 2020). In this regard, the ability to release neurotrophic factors and extend neurites by OE-MSCs was investigated.

The results from the ELISA test indicated that the human OE-MSCs-conditioned medium produced high levels of neurotrophins, NGF, and BDNF. These factors as biological regulators could play important roles in differentiation, myelination, and neuronal growth (Piirsoo et al, 2010). Many studies have used an *in vitro* model to

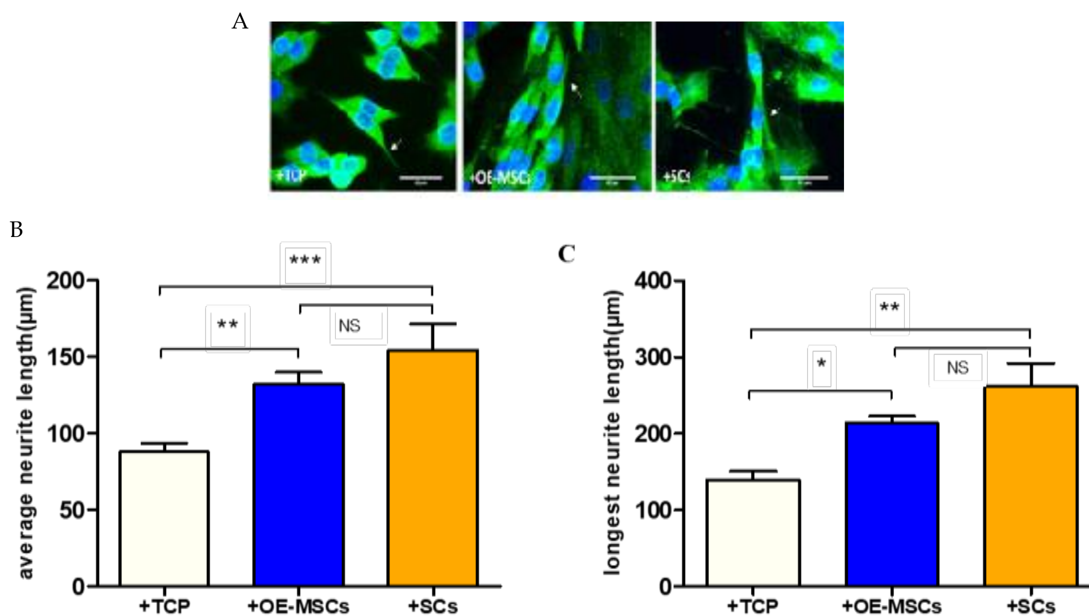

Figure 6. PC12 co-cultured with human olfactory ecto-mesenchymal stem cells and human schwann cells

A) The immunocytochemistry staining for β III-tubulin showed neurites outgrowth from PC12 on tissue culture place, co-cultured with olfactory ecto-mesenchymal stem cells and Schwann cells (arrows) (scale bar=20 µm), B and C) The average neurite length and the longest neurite length were determined in different groups. *P<0.05, **P<0.01, and ***P<0.001.

investigate the function of the derived SCs on the axonal outgrowth and nerve regeneration, and co-cultured them with PC12 cells or primary neurons used as representatives of peripheral neuronal cells (Peng et al., 2011; Xue et al., 2017). After co-culturing OE-MSCs and PC12 cells, it was shown the positive efficacy of these stem cells for neurite outgrowth promotion through intercellular interactions and release of growth factors such as NGF and BDNF.

These findings are in line with a study by Crigler and colleagues who recently showed that human MSCs subpopulations could promote neuritogenesis and neuronal survival by expression of various neuro-regulatory factors, such as NGF and BDNF (Crigler et al., 2006). However, some other studies demonstrated that differentiated MSCs are more beneficial rather than untreated ones in stimulating neurite growth and nerve regeneration (Peng et al., 2011). Although the tendency of human OE-MSCs to spontaneous differentiation into SCs-like cells has been demonstrated *in vitro* models, further studies are required in animal models of peripheral nerve injuries to investigate whether the implantation of these stem cells could improve nerve regeneration.

5. Conclusion

The findings of the present study provided evidence that human OE-MSCs could show properties similar to immature SCs in terms of phenotypic and functional features. It appears that a degree of commitment along SCs lineage already exists for EMSCs migrated to the nasal mucosa. Given the high potential of NC stem cells to produce sufficient SCs for applications in clinics, our results suggested human OE-MSCs as a valuable source of autologous stem cells that can be used for transplantation in peripheral nerve injuries.

Ethical Considerations

Compliance with ethical guidelines

The authors obtained approval from the ethics committee of Iran University of Medical Sciences (Code: 97-02-30-33009) and consent from the patients.

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Authors' contributions

All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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Reference

Achilleos, A., & Trainor, P. A. (2012). Neural crest stem cells: Discovery, properties and potential for therapy. *Cell Research, 22*(2), 288–304. [PMID] [PMCID]

Alvites, R. D., Branquinho, M. V., Caseiro, A. R., Amorim, I., Santos Pedrosa, S., & Rêma, A., et al. (2020). Rat Olfactory Mucosa Mesenchymal Stem/Stromal Cells (OM-MSCs): A characterization study. *International Journal of Cell Biology, 2020*, 2938258. [PMID] [PMCID]

Bagher, Z., Ebrahimi-Barough, S., Azami, M., Mirzadeh, H., Soleimani, M., & Ai, J., et al. (2015). Induction of human umbilical Wharton's jelly-derived mesenchymal stem cells toward motor neuron-like cells. *In Vitro Cellular & Developmental Biology. Animal, 51*(9), 987–994. [PMID]

BBiagiotti, T., D'Amico, M., Marzi, I., Di Gennaro, P., Arcangeli, A., & Wanke, E., et al. (2006). Cell renewing in neuroblastoma: Electrophysiological and immunocytochemical characterization of stem cells and derivatives. *Stem Cells, 24*(2), 443-453. [PMID]

Boerboom, A., Dion, V., Chariot, A., & Franzen, R. (2017). Molecular mechanisms involved in schwann cell plasticity. *Frontiers in Molecular Neuroscience, 10*, 38. [PMID] [PMCID]

Caddick, J., Kingham, P. J., Gardiner, N. J., Wiberg, M., & Terenghi, G. (2006). Phenotypic and functional characteristics of mesenchymal stem cells differentiated along a Schwann cell lineage. *Glia, 54*(8), 840-849. [PMID]

Chen, Q., Zhang, Z., Liu, J., He, Q., Zhou, Y., & Shao, G., et al. (2015). A fibrin matrix promotes the differentiation of EMSCs isolated from nasal respiratory mucosa to myelinating phenotypical Schwann-like cells. *Molecules and Cells, 38*(3), 221–228. [PMID]

Crigler, L., Robey, R. C., Asawachaicharn, A., Gaupp, D., & Phinney, D. G. (2006). Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Experimental Neurology, 198*(1), 54–64. [PMID]

Delorme, B., Nivet, E., Gaillard, J., Haupl, T., Ringe, J., Deveze, A., Feron, F. (2010). The human nose harbors a niche of olfactory ectomesenchymal stem cells displaying neurogenic and osteogenic properties. Stem Cells Dev, 19(6), 853-866. [PMID]

Deng, M. J., Jin, Y., Shi, J. N., Lu, H. B., Liu, Y., & He, D. W., et al. (2004). Multilineage differentiation of ectomesenchymal cells isolated from the first branchial arch. *Tissue Engineering, 10*(9-10), 1597–1606. [PMID]

Duan, D., & Lu, M. (2015). Olfactory mucosa: A rich source of cell therapy for central nervous system repair. *Reviews in The Neurosciences, 26*(3), 281–293. [PMID]

Fu, X., Tong, Z., Li, Q., Niu, Q., Zhang, Z., & Tong, X., et al. (2016). Induction of adipose-derived stem cells into Schwann-like cells and observation of Schwann-like cell proliferation. *Molecular Medicine Reports, 14*(2), 1187–1193. [PMID]

Haratizadeh, S., Nazm Bojnordi, M., Niapour, A., Bakhtiari, M., & Ghasemi Hamidabadi, H. (2016). [Improvement of neuroglial differentiation from human dental pulp stem cells using CSF (Persian)]. *Journal of Mazandaran University of Medical Sciences, 26*(140), 1-14. [Link]

Hassanzadeh, S., Jalessi, M., Jameie, S. B., Khanmohammadi, M., Bagher, Z., & Namjoo, Z., et al. (2021). More attention on glial cells to have better recovery after spinal cord injury. *Biochemistry and Biophysics Reports, 25,* 100905. [PMID]

Hunt, D. P., Morris, P. N., Sterling, J., Anderson, J. A., Joannides, A., & Jahoda, C., et al. (2008). A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin. *Stem Cells (Dayton, Ohio), 26*(1), 163–172.[PMID]

Hussain, G., Wang, J., Rasul, A., Anwar, H., Qasim, M., & Zafar, S., et al. (2020). Current status of therapeutic approaches against peripheral nerve injuries: A detailed story from injury to recovery. *International Journal of Biological Sciences, 16*(1), 116–134 [PMID]

Jessen, K. R., & Mirsky, R. (2019). Schwann cell precursors; Multipotent glial cells in embryonic nerves. *Frontiers in Molecular Neuroscience, 12,* 69. [PMID]

Jessen, K. R., & Mirsky, R. (2019). The success and failure of the schwann cell response to nerve injury. *Frontiers in Cellular Neuroscience, 13,* 33. [PMID]

Jiang, L., Jones, S., & Jia, X. (2017). Stem cell transplantation for peripheral nerve regeneration: Current options and opportunities. *International Journal of Molecular Sciences, 18*(1), 94. [PMID]

Karimi, S., Bagher, Z., Najmoddin, N., Simorgh, S., & Pezeshki-Modaress, M. (2021). Alginate-magnetic short nanofibers 3D composite hydrogel enhances the encapsulated human olfactory mucosa stem cells bioactivity for potential nerve regeneration application. *International Journal of Biological Macromolecules, 167,* 796–806. [PMID]

Kingham, P. J., Kalbermatten, D. F., Mahay, D., Armstrong, S. J., Wiberg, M., & Terenghi, G. (2007). Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. *Experimental Neurology, 207*(2), 267-274. [PMID]

Lin, Y., Yan, Z., Liu, L., Qiao, J., Jing, W., & Wu, L., et al. (2006). Proliferation and pluripotency potential of ectomesenchymal cells derived from first branchial arch. *Cell Proliferation, 39*(2), 79–92. [PMID]

Liu, Q., Spusta, S. C., Mi, R., Lassiter, R. N., Stark, M. R., & Höke, A., et al. (2012). Human neural crest stem cells derived from human ESCs and induced pluripotent stem cells: Induction, maintenance, and differentiation into functional schwann cells. *Stem Cells Translational Medicine, 1*(4), 266–278. [PMID]

López-Cebral, R., Silva-Correia, J., Reis, R. L., Silva, T. H., & Oliveira, J. M. (2017). Peripheral nerve injury: Current challenges, conventional treatment approaches, and new trends in biomaterials-based regenerative strategies. *ACS Biomaterials Science & Engineering, 3*(12), 3098–3122. [PMID]

Lotfi, L., Khakbiz, M., Moosazadeh Moghaddam, M., & Bonakdar, S. (2019). A biomaterials approach to Schwann cell development in neural tissue engineering. *Journal of Biomedical Materials Research. Part A, 107*(11), 2425–2446. [PMID]

Mehrotra, P., Tseropoulos, G., Bronner, M. E., & Andreadis, S. T. (2020). Adult tissue-derived neural crest-like stem cells: Sources, regulatory networks, and translational potential. *Stem Cells Translational Medicine, 9*(3), 328–341. [PMID] [PMCID]

Mirsky, R., Woodhoo, A., Parkinson, D. B., Arthur-Farraj, P., Bhaskaran, A., & Jessen, K. R. (2008). Novel signals controlling embryonic Schwann cell development, myelination and dedifferentiation. *Journal of the Peripheral Nervous System: JPNS, 13*(2), 122–135. [PMID]

Namjoo, Z., Moradi, F., Aryanpour, R., Piryaei, A., Joghataei, M. T., & Abbasi, Y., et al. (2018). Combined effects of rat Schwann cells and 17β-estradiol in a spinal cord injury model. *Metabolic Brain Disease, 33*(4), 1229–1242. [PMID]

Nie, X., Zhang, Y. J., Tian, W. D., Jiang, M., Dong, R., & Chen, J. W., et al. (2007). Improvement of peripheral nerve regeneration by a tissue-engineered nerve filled with ectomesenchymal stem cells. *International Journal of Oral and Maxillofacial Surgery, 36*(1), 32–38. [PMID]

Nocera, G., & Jacob, C. (2020). Mechanisms of Schwann cell plasticity involved in peripheral nerve repair after injury. *Cellular and Molecular Life Sciences: CMLS, 77*(20), 3977–3989. [PMID] [PMCID]

Paino, F., Ricci, G., De Rosa, A., D'Aquino, R., Laino, L., & Pirozzi, G., et al. (2010). Ecto-mesenchymal stem cells from dental pulp are committed to differentiate into active melanocytes. *European cells & Materials, 20,* 295–305. [PMID]

Peng, J., Wang, Y., Zhang, L., Zhao, B., Zhao, Z., & Chen, J., et al. (2011). Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth in vitro. *Brain Research Bulletin, 84*(3), 235–243. [PMID]

Petrova, E. (2015). Injured nerve regeneration using cell-based therapies: Current challenges. *Acta Naturae, 7*(3), 38–47. [DOI:10.32607/20758251-2015-7-3-38-47]

Piirsoo, M., Kaljas, A., Tamm, K., & Timmusk, T. (2010). Expression of NGF and GDNF family members and their receptors during peripheral nerve development and differentiation of Schwann cells in vitro. *Neuroscience Letters, 469*(1), 135–140. [PMID]

Pisciotta, A., Bertoni, L., Vallarola, A., Bertani, G., Mecugni, D., & Carnevale, G. (2020). Neural crest derived stem cells from dental pulp and tooth-associated stem cells for peripheral nerve regeneration. *Neural Regeneration Research, 15*(3), 373–381.[PMID]

Resch, A., Wolf, S., Mann, A., Weiss, T., Stetco, A. L., & Radtke, C. (2018). Co-Culturing human adipose derived stem cells and schwann cells on spider silk-A new approach as prerequisite for enhanced nerve regeneration. *International Journal of Molecular Sciences, 20*(1), 71. [PMID]

Rui, K., Zhang, Z., Tian, J., Lin, X., Wang, X., & Ma, J., et al.(2016). Olfactory ecto-mesenchymal stem cells possess immunoregulatory function and suppress autoimmune arthritis. *Cellular & Molecular Immunology, 13*(3), 401–408. [PMID]

Shafiee, A., Kabiri, M., Ahmadbeigi, N., Yazdani, S. O., Mojtahed, M., & Amanpour, S., et al (2011). Nasal septum-derived multipotent progenitors: A potent source for stem cell-based regenerative medicine. *Stem Cells and Development, 20*(12), 2077–2091. [PMID]

Simorgh, S., Alizadeh, R., Shabani, R., Karimzadeh, F., Seidkhani, E., Majidpoor, J., et al (2021). Olfactory mucosa stem cells delivery via nasal route: A simple way for the treatment of Parkinson disease. *Neurotoxicity Research, 39*(3), 598–608. [PMID]

Simorgh, S., Bagher, Z., Farhadi, M., Kamrava, S. K., Boroujeni, M. E., & Namjoo, Z., et al. (2021). Magnetic targeting of human olfactory mucosa stem cells following intranasal administration: a novel approach to Parkinson's disease treatment. *Molecular Neurobiology, 58*(8), 3835–3847. [PMID]

Solis-Castro, O. O., Boissonade, F. M., & Rivolta, M. N. (2020). Establishment and neural differentiation of neural crest-derived stem cells from human dental pulp in serum-free conditions. *Stem Cells Translational Medicine, 9*(11), 1462–1476. [PMID] [PMCID]

Veron, A. D., Bienboire-Frosini, C., Feron, F., Codecasa, E., Deveze, A., & Royer, D., et al. (2018). Isolation and characterization of olfactory ecto-mesenchymal stem cells from eight mammalian genera. *BMC Veterinary Research, 14*(1), 17.[PMID]

Veron, A. D., Bienboire-Frosini, C., Girard, S. D., Sadelli, K., Stamegna, J. C., & Khrestchatisky, M., et al. (2018). Syngeneic transplantation of olfactory ectomesenchymal stem cells restores learning and memory abilities in a rat model of global cerebral ischemia. *Stem Cells International, 2018,* 2683969. [PMID]

Woodhoo, A., & Sommer, L. (2008). Development of the Schwann cell lineage: From the neural crest to the myelinated nerve. *Glia, 56*(14), 1481–1490. [PMID]

Xue, J., Yang, J., O'Connor, D. M., Zhu, C., Huo, D., & Boulis, N. M., et al. (2017). Differentiation of bone marrow stem cells into Schwann cells for the promotion of neurite outgrowth on electrospun fibers. *ACS Applied Materials & Interfaces, 9*(14), 12299–12310. [PMID]

Zaminy, A., Shokrgozar, M. A., Sadeghi, Y., Noroozian, M., Heidari, M. H., & Piryaei, A. (2013). Mesenchymal stem cells as an alternative for Schwann cells in rat spinal cord injury. *Iranian Biomedical Journal, 17*(3), 113–122. [PMID]

Zhang, J., Gao, X., Zou, H., Liu, J., & Zhang, Z. (2015). Rat nasal respiratory mucosa-derived ectomesenchymal stem cells differentiate into Schwann-like cells promoting the differentiation of PC12 cells and forming myelin in vitro. *Stem Cells International, 2015,* 328957. [PMID]

Zhang, Z., He, Q., Deng, W., Chen, Q., Hu, X., & Gong, A., et al. (2015). Nasal ectomesenchymal stem cells: multi-lineage differentiation and transformation effects on fibrin gels. *Biomaterials, 49,* 57–67. [PMID]

Duraikannu, A., Krishnan, A., Chandrasekhar, A., & Zochodne, D. W. (2019). Beyond trophic factors: Exploiting the intrinsic regenerative properties of adult neurons. *Frontiers in Cellular Neuroscience, 13,* 128. [PMID]